

Production of High Concentrations of Ethanol from Inulin by Simultaneous Saccharification and Fermentation Using *Aspergillus niger* and *Saccharomyces cerevisiae*

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Pure nonhydrolyzed inulin was directly converted to ethanol in a simultaneous saccharification and fermentation process. An inulinase-hyperproducing mutant, *Aspergillus niger* 817, was grown in a submerged culture at 30°C for 5 days. The inulin-digestive liquid culture (150 ml) was supplemented with 45 g of inulin, 0.45 g of (NH₄)₂SO₄, and 0.15 g of KH₂PO₄. The medium (pH 5.0) was inoculated with an ethanol-tolerant strain, *Saccharomyces cerevisiae* 1200, and fermentation was conducted at 30°C. An additional 20 g of inulin was added to the culture after 15 h of fermentation. *S. cerevisiae* 1200 utilized 99% of the 65 g of inulin during the fermentation, and produced 20.4 and 21.0% (vol/vol) ethanol from chicory and dahlia inulins, respectively, within 3 days of fermentation. The maximum volumetric productivities of ethanol were 6.2 and 6.0 g/liter/h for chicory and dahlia inulins, respectively. The conversion efficiency of inulin to ethanol was 83 to 84% of the theoretical ethanol yield.

Inulin is a polyfructan, consisting of linear β -2,1-linked polyfructose chains terminated by a glucose residue attached through a sucrose type linkage (16). This polymer is a reserve carbohydrate in the roots and tubers of plants like Jerusalem artichoke, chicory, or dahlia. Such inulin sources have recently received renewed attention as a potential feedstock for fuel ethanol production (1-3, 8). The direct fermentation of Jerusalem artichoke extracts into ethanol by several inulinase-producing yeasts such as *Kluyveromyces fragilis*, *Kluyveromyces marxianus*, and *Saccharomyces rosei* (1, 3, 8) has been studied. Another approach involves a two-step process, in which enzymatically hydrolyzed Jerusalem artichoke extracts were used with the bacterium *Zymomonas mobilis* for subsequent fermentation to ethanol (2).

Microbial inulinases (2,1- β -D-fructan fructanohydrolase [EC 3.2.1.7]) are usually inducible and exo-acting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose) (16). Nakamura et al. (11) isolated *Aspergillus niger* 12, which produced high levels of inulinases irrespective of the carbon source. Strain 12 secreted both exo- and endoinulinases extracellularly (12-14). The purified endoinulinase P-III hydrolyzed inulin to inulotriose, inulotetraose, and inulopentaose (12, 14). Nakamura et al. generated a mutant, strain 817, from strain 12 by γ -irradiation with ⁶⁰Co which formed even higher levels of inulinases than the wild-type strain 12, as shown in this article.

Hayashida et al. (7) have reported the production of 20.1% (vol/vol) ethanol from raw ground corn in 5 days by the simultaneous saccharification and fermentation process. In this single-step process, *Aspergillus awamori* var. *kawachi* and *Saccharomyces* sp. strain WY2 were used concurrently for their raw starch digestion and ethanol production, respectively. In the present study, the availability of *A. niger* 817 allowed us to develop and evaluate the simultaneous

saccharification and fermentation of inulin to ethanol in combination with *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Organisms. *A. niger* 817 used in the present study was a mutant derived from inulinase-hyperproducing *A. niger* 12 (11). The stock cultures were maintained on agar slants (see Table 1). A wild-type strain, *S. cerevisiae* 1200 (see below for identification), was chosen for ethanol fermentation because of its high ethanol tolerance as described below. The ethanol-tolerant strain 1200 was originally isolated from a fermenting mash for shochu (a Japanese spirit) manufactured in Kuma, Japan. It was maintained on slants of YPD agar medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar). Identification of strain 1200 was performed by standard methods used in yeast taxonomy (17).

Substrates. Pure nonhydrolyzed inulin was used as a model substrate for the simultaneous saccharification and fermentation experiments. Commercial preparations of pure inulin derived from chicory roots and dahlia tubers were obtained from Sigma Chemical Co., St. Louis, Mo. The average size of the polyfructans varies with the source and the length of storage. The polyfructans with a degree of polymerization of at least 30 are referred to as inulin (16). The average degree of polymerization was calculated as the ratio of glucose to fructose on an acid-hydrolyzed sample. The values of the average degree of polymerization were 33.6 in chicory inulin and 40.3 in dahlia inulin.

Assay of inulinase and invertase activities. Exoinulinase P-II from *A. niger* 12 hydrolyzed sucrose and raffinose in addition to inulin, while endoinulinase P-III did not hydrolyze either sucrose or raffinose (14). To characterize inulinases, inulinase activity is commonly compared with the invertase activity displayed by the same enzyme preparation. The extracellular inulinase and invertase activities in culture supernatants were assayed by measuring reducing sugars released from inulin and sucrose, respectively, as described by Nakamura and Hoashi (10). One unit of inulinase activity was defined as the amount of enzyme in 1 ml

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TABLE 1. Composition of culture media for maintenance and inulinase production of *A. niger* 817^a

Ingredient	Amt (g/liter) in:	
	Slant culture	Liquid culture
Sucrose	10	30
Peptone	5	
Sucrose fatty acid ester ^b		5
Corn steep liquor		15
NH ₄ H ₂ PO ₄		12
(NH ₄) ₂ HPO ₄	3	
K ₂ HPO ₄	1	
KCl	0.5	0.7
MgSO ₄ · 7H ₂ O	0.5	0.5
FeSO ₄ · 7H ₂ O	0.01	0.01
Agar	20	

^a The initial pH values of media for slant and liquid cultures were adjusted to pH 5.5 and 4.5, respectively, by using 1 N HCl or 1 N NaOH before sterilization.

^b Ryoto sugar ester S-770 containing 70% stearic acid was used and obtained from Mitsubishi-Kasei Foods Co., Tokyo, Japan.

that liberates 1 μ mol of fructose equivalent from inulin per min. One unit of invertase activity was defined as the amount of enzyme in 1 ml that catalyzes the hydrolysis of 1 μ mol of sucrose per min.

Culture conditions for inulinase production by *A. niger* 817.

The culture medium for inulinase production (Table 1) was a modification of the basal medium B of Nakamura et al. (11) and contained sucrose as the carbon source and sucrose fatty acid ester as a surfactant to release readily intracellular inulinases. Portions (120 ml) of the culture medium were dispensed into 500-ml Erlenmeyer flasks, autoclaved at 121°C for 20 min, and inoculated with spores from slant cultures of strain 817. A total of 18 cultures were incubated on a gyratory shaker at 30°C and 140 rpm for 8 days. For maintaining the desired pH, calcium carbonate was added to give a concentration of 1% (wt/vol) after 24 h of incubation. The contents of duplicate flasks were analyzed daily for inulinase, invertase, reducing sugar, total sugar, pH, and biomass. The 5-day-old liquid culture was employed in subsequent fermentation experiments.

Fermentation tests. Fermentation tests for determining the maximum concentration of ethanol attained by *S. cerevisiae* 1200 were carried out in fed-batch cultures with sucrose at 30°C as described previously (4). Either whole liquid culture or mycelium-free culture supernatant of *A. niger* 817 (150 ml) was supplemented with 30 g of sucrose, 0.45 g of (NH₄)₂SO₄, and 0.15 g of KH₂PO₄, and the two were compared for use as a fermentation medium. The initial pH of the media was 5.0, and the pH was not controlled during fermentation. For the inoculum, *S. cerevisiae* cells from a fresh slant culture were suspended in 100 ml of a liquid medium composed of 1% yeast extract, 2% peptone, and 20% glucose in a 500-ml shake flask. Inoculum cultures were grown on a gyratory shaker at 30°C and 140 rpm for 24 h. *S. cerevisiae* cells were collected by centrifugation and resuspended in the 150-ml medium to give 10⁸ cells per ml of medium in 250-ml culture bottles fitted with fermentation bungs. These fermentation bungs were partially filled with concentrated sulfuric acid and permitted CO₂ to be released from the culture vessel and maintain anaerobic conditions. Each culture was incubated statically at 30°C until fermentation ceased. Fermentations were monitored by CO₂ evolution. The weight loss of the

culture was used to calculate residual sucrose. The sucrose consumption was calculated assuming 90% of theoretical conversion of sucrose to ethanol plus CO₂ as follows: amount of CO₂ evolved (in grams) \times 2.16 = amount of sucrose fermented (in grams). Solid sucrose in small increments was fed sequentially to the culture to maintain the sucrose concentration at subinhibitory levels.

Simultaneous saccharification and fermentation procedures.

The saccharification and fermentation of inulin proceeded simultaneously within the one vessel, essentially as described for ethanol fermentation of raw ground corn (7). The whole liquid culture (150 ml) of *A. niger* 817 was dispensed into 500-ml culture bottles fitted with fermentation bungs and supplemented with 45 g of inulin, 0.45 g of (NH₄)₂SO₄, and 0.15 g of KH₂PO₄ for use as a fermentation medium without sterilization. The medium (pH 5.0) was inoculated with *S. cerevisiae* cells from the inoculum culture as described above for fermentation tests, and each culture was incubated at 30°C for 4 days. Since pure inulin is almost insoluble in water at room temperature, the initial broth in which 45 g of inulin was added to the 150 ml of fungal liquid culture gave a highly viscous slurry. While the viscosity decreased rapidly, probably because of the inulin solubilization by endoinulinases, the gas evolution carried the solid matter to the surface. Frequent shaking was necessary to keep the solid matter submerged in the liquid in an attempt to secure complete fermentation during the first 15 h of fermentation. After this period, these cultures were permitted to grow statically. Additional inulin (20 g) was added to the culture after 15 h of fermentation.

Analytical methods. (i) **Inulinase production.** Reducing sugars were determined by the dinitrosalicylic acid method (9). Total sugars were estimated as reducing sugars after acid hydrolysis (0.1 N HCl, 30 min, 100°C). Biomass concentration was determined by harvesting the mycelial pellets by filtration and freeze-drying them to a constant weight. The dry weight was expressed as grams per liter of the fungal culture.

(ii) **Ethanol fermentation.** During fermentation, samples (1 ml) were periodically withdrawn and centrifuged at 4°C for 5 min at 5,000 \times g to remove cells. Supernatant fluid was used for ethanol analysis (see below). In the fermentation experiments with inulin, reducing sugars in the supernatant were also determined as described above. A standard curve for this colorimetric assay was constructed by using fructose as the standard. At the end of the fermentation, total sugars were determined as reducing sugars after acid hydrolysis (1 N HCl, 60 min, 100°C). The final titratable acidity was expressed as the total volume of 0.1 N NaOH added to 5 ml of culture supernatant until the pH, measured by a pH meter, reached 7.0.

Ethanol concentration. The ethanol concentration was determined by using a Shimadzu gas chromatograph (model GC-14) with a flame ionization detector. The glass column (2.1 m by 3 mm), packed with 5% Thermon 1000 on Sunpak-A (Shimadzu Co., Kyoto, Japan), was maintained at 150°C. N₂ was used as a carrier gas at a flow rate of 30 ml/min. Ethanol was quantified by using isopropanol as the internal standard.

Calculation of kinetic and yield parameters. Maximum volumetric productivities of ethanol were estimated from the steepest slope of each fermentation profile. The amount of free fructose liberated from inulin was calculated by using the following formula: amount of fructose (in grams) \times 0.90 = amount of inulin (in grams). The theoretical ethanol yield was calculated assuming the complete conversion of 180 g of

fructose (1 mol) to 92 g of ethanol (2 mol). This value was then used to calculate the conversion efficiency (percent) of inulin to ethanol in the experimental culture on the basis of the sugar consumed.

RESULTS

Morphological and physiological characteristics of *S. cerevisiae* 1200. The vegetative cells of strain 1200 were globose to ellipsoidal, 4.6 to 5.3 by 6.3 to 10.1 μm in size, and reproduced by multilateral budding. Pseudomycelium was formed on potato-glucose agar. Sporulation was observed on acetate agar, and two to four ascospores were produced per ascus. Strain 1200 was unable to utilize nitrate, ethylamine, or cadaverine and was sensitive to 100 ppm of cycloheximide. It was able to ferment glucose, galactose, sucrose, maltose, and raffinose, but was unable to ferment melibiose. On the basis of the morphological and physiological properties, the strain was identified as *S. cerevisiae* (18).

Time course of inulinase production by *A. niger* 817. The time courses of inulinase and invertase activities, residual sugars, pH, and biomass were determined in shake flask cultures of *A. niger* 817 at 30°C (Fig. 1). Inulinase activity reached a maximum of 48.4 U/ml after 7 days, while invertase activity increased gradually to 14.6 U/ml. The liquid culture was thus characterized by a much higher activity on inulin than on sucrose. This is an indication that endoinulinases are predominant. Reducing sugars liberated from sucrose increased between 24 and 48 h because of the invertase activity. Nearly all of the sugars were exhausted in 6 days. The pH value remained nearly constant (4.5 to 5.1) throughout the culture period. Strain 817 grew well to a cell density of 22.9 g/liter.

Ethanol tolerance and volumetric ethanol productivity of *S. cerevisiae* 1200 in fed-batch fermentation. Prior to inulin fermentation studies, *S. cerevisiae* 1200 was evaluated for its ethanol tolerance in a fed-batch fermentation of sucrose, using either whole liquid culture or culture supernatant of *A. niger* 817 as the fermentation media (Fig. 2). A total of 64 g of sucrose was added sequentially to the mycelium-containing culture during the ethanol fermentation, and the final ethanol concentration of 21.3% (vol/vol) was obtained in 5 days. In the mycelium-free culture, however, a lower ethanol concentration of 17.8% (vol/vol) was produced from a total of 55 g of sucrose in 6 days. Volumetric ethanol productivities reached maximum values of 6.6 and 4.8 g/liter/h for mycelium-containing and mycelium-free cultures, respectively.

Simultaneous saccharification and fermentation of pure nonhydrolyzed inulin. Since the presence of fungal mycelia in fed-batch fermentation was responsible for the improved ethanol tolerance of *S. cerevisiae* 1200, the 5-day-old liquid culture of *A. niger* 817 was used directly as the inulinase source without filtration in the simultaneous saccharification and fermentation of inulin. The inulinase and invertase activities in the supernatant of the liquid culture were 44.8 and 5.91 U/ml, respectively. The pH was 4.8, and the biomass concentration was 18.2 g/liter. On the basis of the amount of sucrose (64 g) fed to the mycelium-containing culture (Fig. 2A), a total of 65 g of pure nonhydrolyzed inulin from chicory roots and dahlia tubers was added to the 150 ml of the fungal liquid culture as the substrate. When either chicory or dahlia inulin was used as the substrate, comparable fermentation profiles were obtained (Fig. 3). Fermentations were completed in 3 days, and maximum ethanol concentrations of 20.4 and 21.0% (vol/vol) were obtained

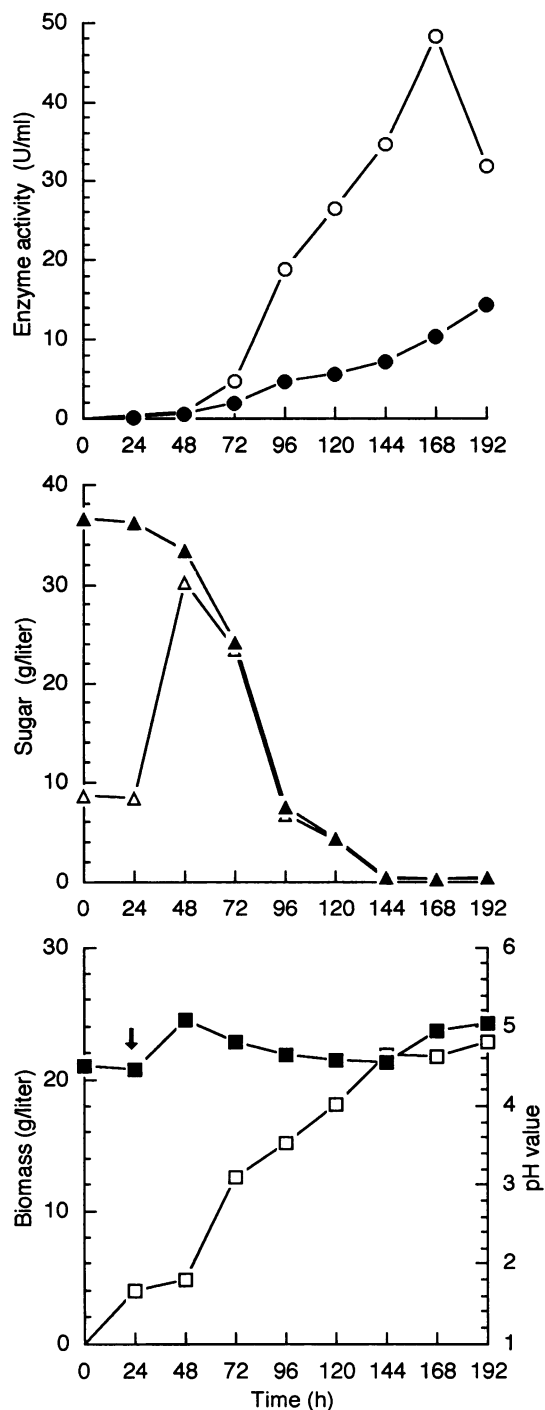


FIG. 1. Time course of inulinase production in shake flask cultures of *A. niger* 817. This experiment was conducted in 18 Erlenmeyer flasks containing 120 ml of liquid medium and incubated with shaking at 30°C. The contents of duplicate flasks were used daily for analyses. The arrow indicates the addition of 1.2 g of calcium carbonate after 24 h of incubation. Symbols: ○, inulinase; ●, invertase; △, reducing sugar; ▲, total sugar; □, biomass; ■, pH.

from chicory and dahlia inulins, respectively. No significant bacterial growth was observed microscopically during the fermentation.

The time course of residual reducing sugars (Fig. 3) shows

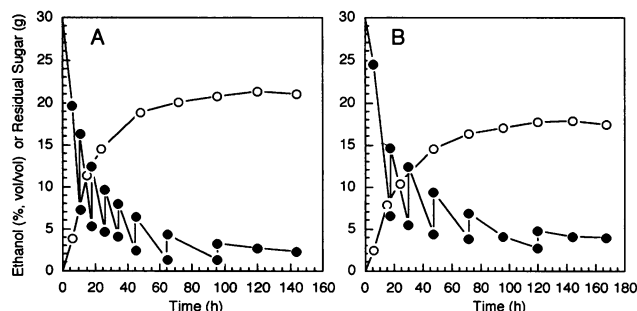


FIG. 2. Ethanol production by *S. cerevisiae* 1200 in fed-batch fermentation of sucrose. Either whole liquid culture (A) or culture supernatant (B) of *A. niger* 817 (150 ml) was supplemented with 30 g of sucrose and inoculated with the *S. cerevisiae* 1200 to give 10^8 cells per ml. Fermentations were carried out at 30°C. Solid sucrose was fed sequentially. Symbols: ○, ethanol; ●, residual sugar.

a rapid increase in reducing sugar concentration to 16 to 20% (wt/vol) during the first 6 h of fermentation. A second increase in reducing sugar concentration was observed between 15 and 24 h, since an additional 20 g of inulin was added to the culture after 15 h of fermentation. Thus, sufficient fermentable sugars released from inulin by the extracellular inulinases of *A. niger* 817 allowed the rapid fermentation by *S. cerevisiae* 1200 similar to the above fed-batch fermentation of sucrose in the presence of fungal mycelia.

Table 2 summarizes the fermentation parameters of this process. Volumetric productivities of ethanol reached maximum values of 6.2 and 6.0 g/liter/h for chicory and dahlia inulins, respectively, between 6 and 15 h. Analytical data of final residual sugars showed that *S. cerevisiae* 1200 utilized 99% of the 65 g of inulin saccharified almost completely by *A. niger* 817 during the fermentation. Ethanol yields were 0.42 and 0.43 g of ethanol per g of the sugar consumed (83 and 84% of the theoretical yield), respectively.

DISCUSSION

Table 3 provides a comparison of several batch fermentations for ethanol production with our results. Among the inulinase-producing yeasts studied, *K. marxianus* gave the

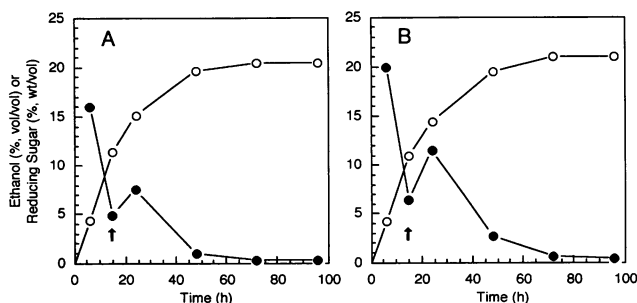


FIG. 3. Simultaneous saccharification and fermentation of inulin to ethanol by *A. niger* 817 and *S. cerevisiae* 1200. Whole liquid culture (150 ml) of the fungal strain 817 was supplemented with 45 g of pure inulin from either chicory (A) or dahlia (B) and inoculated with the *S. cerevisiae* 1200 to give 10^8 cells per ml. Fermentations were carried out at 30°C. The arrows indicate further addition of 20 g of inulin after 15 h of fermentation. Symbols: ○, ethanol; ●, reducing sugar.

TABLE 2. Fermentation parameters in simultaneous saccharification and fermentation of inulin to ethanol by *A. niger* 817 and *S. cerevisiae* 1200

Fermentation parameters	Inulin from:	
	Chicory	Dahlia
Broth volume (ml) ^a	183	182
Total amount of inulin (g) ^b	65	65
Final ethanol concn (g/liter)	163	168
Ethanol yield (g/g of sugar)	0.42	0.43
Conversion efficiency (%) ^c	83	84
Maximum volumetric productivity of ethanol (g/liter/h) ^d	6.2	6.0
Final total sugars (g/liter)	3.22	2.86
Final reducing sugars (g/liter)	2.70	2.84
Final titratable acidity (ml)	5.7	5.6
Final pH	4.6	4.6
Final biomass concn (g/liter) ^e	28.9	28.8

^a Broth volume was measured at the end of fermentation and corrected by adding the total volume of samples removed periodically for chemical analysis.

^b The 65 g of inulin is equivalent to 72.2 g of fructose.

^c Percentage of the theoretical ethanol yield (0.51 g of ethanol per g of fructose).

^d Computed from the slope of fermentation profile between 6 and 15 h.

^e Combined cell biomass of *A. niger* 817 plus *S. cerevisiae* 1200.

best results, producing 7.7% (vol/vol) ethanol directly from Jerusalem artichoke extracts after 4 days of fermentation (3). In actual Jerusalem artichoke extracts, it is expected that a partial hydrolysis of the polyfructans occurred during the extraction process. Pure inulin was more difficult for inulinase-producing yeasts to break down into fermentable sugars (8). In this study, much higher final ethanol concentrations and maximum volumetric productivities of ethanol were attained by the simultaneous saccharification and fermentation of pure inulin. Comparable high concentrations of ethanol (>20%, vol/vol) were previously obtained from raw ground corn by a similar process (7). Such direct fermentation processes eliminate the separate hydrolysis step of inulin, which was necessary for the fermentation with *Z. mobilis* (2). *Z. mobilis* converted rapidly and efficiently the enzymatically hydrolyzed Jerusalem artichoke extracts to ethanol, although final ethanol concentrations remained low.

This is the first description of the concurrent use of *A. niger* and *S. cerevisiae* for the direct conversion of pure inulin to ethanol. A mutant strain, *A. niger* 817, produced two- or three-times-higher levels of extracellular inulinases than the wild-type strain 12 (11). Purification and characterization of inulinases from strain 817 (unpublished data) confirmed the coexistence of exo- and endoinulinases in the liquid culture. The concerted action of exo- and endoinulinases on inulin might allow more effective hydrolysis in contrast with that of inulinase-producing yeasts in which only exoinulinases were present. While the saccharification of high concentrations of inulin by the fungal inulinases proceeds, the rapid yeast fermentation of the liberated sugars to ethanol kept the sugar concentration low enough to prevent feedback inhibition of inulinase activity. In addition to the high inulinase activity, the presence of the fungal mycelia enhanced the volumetric productivity of ethanol and the ethanol tolerance of *S. cerevisiae* 1200 as expected, because the proteolipids present in the fungal mycelia acted as a high-concentration ethanol-producing factor (6). When grown with the fungal proteolipids under anaerobic conditions, *S. cerevisiae* cells were enriched with the exogenous

TABLE 3. Comparison of several batch fermentations for ethanol production

Organism	Substrate	Process type ^a	Ethanol concn (%, vol/vol)	Volumetric productivity (g/liter/h)	Conversion efficiency (%)	Reference
<i>K. marxianus</i>	Jerusalem artichoke extract	One-step	7.7	1.5 ^b	89 ^b	3
<i>Z. mobilis</i>	Jerusalem artichoke extract	Two-step	12.5	3.4 ^b	92	2
<i>A. awamori</i> var. <i>kawachi</i> and <i>Saccharomyces</i> sp.	Raw ground corn	SSF	20.1	3.1 ^b	NA ^c	7
<i>A. niger</i> and <i>S. cerevisiae</i>	Pure inulin	SSF	20.4–21.0	6.0–6.2	83–84	This study

^a Processes: one-step, the direct conversion of Jerusalem artichoke extract to ethanol by the inulinase-producing *K. marxianus*; two-step, the enzymatic hydrolysis of Jerusalem artichoke extract and the subsequent fermentation; SSF, simultaneous saccharification and fermentation.

^b The value is not explicitly given in the article but is derived from other data or graphics.

^c NA, not available.

highly unsaturated phospholipids and sterol-unsaturated fatty acid esters and consequently acquired enhanced fermentative activity and ethanol tolerance, respectively (5, 15). Thus, the synergistic combination of selected strains of *A. niger* and *S. cerevisiae* resulted in the rapid production of high concentrations of 20 to 21% (vol/vol) ethanol from inulin within 3 days at 30°C.

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